National Institute of Dental and Craniofacial Research
Division of Intramural Research

Annual Report Summary

1999

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Fiscal Year 1999

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Introduction and Overview

Henning Birkedal-Hansen, Scientific Director

INTRODUCTION

The Division of Intramural Research, NIDCR, experienced a very active and productive FY 1999. While the individual Branch reports summarize the Division's research activities during the past fiscal year a number of other areas also deserve mention.

Scientific Productivity

In FY 1999 the Division's scientific productivity and quality continued at a remarkably high level and the impact of the contributions were broad and deep. Each of the seven Branches provided new insights into the rapidly growing biomedical research base. The exceptional quality of the science culminated in articles in Cell, Nature, Science, PNAS, as well as other leading biomedical research journals. The Oral Infection and Immunity Branch completed its quadrennial Board of Scientific Counselors review and received highly laudatory comments for its innovative and substantive programs and for its continued scientific progress during the past four years.

As evidence of their scientific contributions, Dr. Yoshihiko Yamada and Dr. Pamela Gehron Robey were selected for appointments to the prestigious NIH Senior Biomedical Research Service (SBRS). Selection into the extremely competitive SBRS represents the highest level of recognition by one's peers as only 120 have been selected at NIH altogether. Drs. Yamada and Robey joined NIDCR scientists Drs. Dennis Torchia, Bruce Baum, Sharon Wahl and Silvio Gutkind who were appointed to the SBRS in previous years.

NIDCR scientists continue to serve frequently as organizers and/or speakers at international scientific meetings and symposia. These activities, as well as other interactions between the Division and the extramural scientific community, are detailed in a separate document titled "Interactions with the Scientific Community."

New and Expanded Programs

The scientific programs were expanded in several areas. The Craniofacial and Skeletal Diseases Branch initiated new protocols in the Clinical Center aimed at redirecting its efforts increasingly toward clinical approaches to human skeletal diseases. The Oral and Pharyngeal Cancer Branch expanded its research program in two new areas. One with the recruitment of Dr. Thomas Bugge, who will conduct research on mouse genetics/animal models of cancer, and the other with the inauguration of a national search for a medical oncologist. These recruitments will allow the Branch to increasingly move its basic research program in a translational and clinical direction. Upon the departure of Dr. Brian O'Connnell, the Gene Therapy and Therapeutics Branch recruited Dr. Jay Chiorini, a virologist, to better meet the needs and demands of the future development of the next generation of viral-derived vectors for gene therapy. The Branch transferred the responsibilities for the Sjogren's clinic from Dr. Philip Fox, who left the NIH to pursue clinical research in private industry, to Dr. Stanley Pilemer. Sjorgren's Syndrome continues to be a significant focus for the Branch.

Clinical Research – Clinical Director

Dr. Anne O'Connell, Acting Clinical Director, returned to her native Ireland in July and following a national search, Dr. Raymond A. Dionne was named to the position of Clinical Director. Dr. Dionne will play a key role in developing and managing the Division's clinical research and training programs. Dr. Dionne's responsibilities will be somewhat different from previous Clinical Directors as the interfacing with the Clinical Center and other ICs during the construction of the new Clinical Center wing, and the adjustments that need to be made in space and resources to accommodate these changes, require a higher level of leadership and detailed involvement than before. In addition, we are beginning to see the results of our planning for greater involvement in clinical and patient oriented research throughout the Division which requires a great amount of guidance and input from the Clinical Director. Finally, the rules and regulations that govern the conduct of clinical research, as well as their proper enforcement, are becoming ever more stringent and require additional oversight and review. All of these responsibilities fall under the venue of the Clinical Director. In addition, the Clinical Director is being asked to develop a multifaceted, multi-disciplinary menu of clinical research training programs primarily, but not exclusively, geared toward individuals with DMD/DDS degrees. The programs will be customized to suit the individual candidate's clinical and scientific background. In addition to a basic core, curricula may take a number of divergent paths spanning from public health dentistry, epidemiology or bench-bedside projects depending on the underlying basic science.

Space and Renovation

The laboratory space in Buildings 30 and 10 has remained essentially unchanged since 1961, the same year that Building 30 opened. The Division has therefore begun phasing in a comprehensive renovation program that will bring the current research space up to modern standards. FY 1999 marked the first full year of renovations in both Building 30 and in Building 10 and the first few laboratories have been completed with truly astounding results. At this time, renovation projects are in progress on virtually every floor of NIDCR space. The renovation has been immensely important for the program as a whole and has greatly enhanced the spirit and morale of the scientists who are now working in space that matches the high quality of their research.

Training

The Office of Education is now fully operational under the leadership of Dr. Sharon Gordon. Dr. Gordon has initiated a number of outreach and training activities that allow the Division to communicate more effectively with the dental, oral and craniofacial research communities. In addition, the Office has greatly facilitated communication within the program and made it possible for us to keep fellows and students apprised of opportunities within the Division and on campus. During the past year, all of the recommendations made by the Fellows Committee under the NIDCR Strategic Plan have been implemented. The Division is very proud of its Office of Education and looks forward to increasing benefits from this office. As one piece of tangible evidence, the Office was able to guide 3 fellows successfully through the NIH loan repayment program competition.

FY 1999 marked the fourth year of the highly successful Dental Student Award, a competitive program that brings 10-12 dental students from across the country to Bethesda to conduct research over the summer in the Division's laboratories and clinics. The program is becoming increasingly competitive and the quality of the applicants is remarkably high.

Administrative Issues

On the administrative front, FY 1999 marked the first full year of complete transition from a Branch-based to a Senior Investigator-based budget system that empowers investigators to independently budget and allocate their own resources. This represents the next step in moving entirely toward project-based funding, which will streamline budget planning, scientific progress review and resource accountability. In addition, the system will simplify reporting to the Board of Scientific Counselors and to the National Advisory Dental Resource Council.

Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada
Hynda Kleinman
Yoshihiko Yamada

CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH
1999

The goals of the Craniofacial Developmental Biology and Regeneration Branch (CDBRB) focus on creating new research breakthroughs to (a) understand the mechanisms of normal and abnormal craniofacial development and function at genetic, molecular, and cell biological levels, (b) discover new genes, biologicals, and biomimetics relevant to diagnosis, repair, and therapy, and (c) develop creative, biologically based methods to replace craniofacial tissues that are defective or damaged. Particular emphasis is placed on the interface between cells and extracellular molecules. Our mission spans the range from basic research to clinical, and from normal development to anomalies, wound healing, cancer, and AIDS. CDBRB researchers are exploring important, fundamental questions in developmental biology and related fields, such as the molecular and cell biological mechanisms of morphogenesis, formation and functions of extracellular matrix and its receptors, signaling from the cell surface to the nucleus for novel gene induction, cellular differentiation, and the mechanisms of cancer cell growth and metastasis. Discoveries and ongoing innovations in this basic research will provide the basis for novel translational and patient-oriented applications. This past year, our researchers continued to generate a variety of exciting research advances and to receive international recognition. We also continued to place high priority on training younger scientists to become independent leaders in academia and industry. In addition, we provided extensive service and citizenship activities on behalf of NIDCR, NIH, and our research fields.

Researchers in the CDBRB have made substantial progress and exciting scientific breakthroughs during the past year, and our annual report bibliography lists 85 publications. Several selected research advances are highlighted below. The project reports from each branch section provide more comprehensive summaries of the major new findings in our Branch.

CDBRB initiated the Oral and Craniofacial Genome Anatomy Project (OC-GAP) to catalogue genes expressed in oral and craniofacial tissues and to discover novel genes important for tooth, oral, and craniofacial development. Previously, CDBRB researchers discovered ameloblastin, a tooth-specific, developmentally regulated gene associated with enamel formation and linked to the congenital disorder amelogenesis imperfecta. This year, we described vinexin, a novel cytoskeletal gene that helps to regulate cell adhesion and spreading. Other new genes currently being identified may be candidates for the identification of genetically linked diseases and disorders of oral and craniofacial tissues, and others may have important roles in developmental or disease processes. Hundreds of novel genes have been identified from mouse embryonic craniofacial or human salivary cDNA subtraction libraries. Novel and known genes showed interesting and quite distinctive mRNA expression patterns in developing tissues or in response to a specific extracellular matrix protein. For example, twenty novel genes are expressed preferentially in early mouse craniofacial tissue, and 10% of 2,200 previously identified mouse genes are expressed in stage-specific patterns during craniofacial development. Unique clones with interesting expression patterns are being characterized further. CDBRB members are Project Officers of a major new contract to be awarded shortly to discover and catalogue expression patterns of human craniofacial genes that are active during early development. All clones and antibodies will continue to be made freely available to dental scientists and other qualified investigators to promote research in the area.

Our knowledge about gene regulation and the extracellular matrix is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has created gene knockout mice to study the biological roles of link protein and perlecan. Link protein-null mutants showed progressive dwarfism, suggesting a critical role for link protein in the maintenance of cartilage structure and function during skeletal development, chondrocyte differentiation, and endochondral ossification. The phenotype of the link protein mutant mice may provide useful clues for the identification of link protein gene defects in human chondrodysplasias. In contrast, perlecan-deficient mice showed abnormal cephalic and skeletal development. The phenotype of perlecan mutant mice is remarkably similar to human thanatophoric dysplasia type I, which is caused by activating mutations in FGFR3. These results suggest the involvement of perlecan in FGF receptor signaling in skeletal development.

Laminin and laminin peptides have been implicated by CDBRB members in angiogenesis, neurite outgrowth, and tumor growth and metastasis. Over 700 overlapping laminin peptides spanning the entire laminin molecule are being tested in a variety of biological processes by the Molecular and Cell Biology Sections. Many of these peptides have cell-type specific effects on cell adhesion, growth, angiogenesis, or salivary gland differentiation; several are highly potent. For example, one peptide can cause tumor cells to metastasize to the liver, and another increases lung colonization by 5-fold. The receptors for both have been identified: the former is a cell surface heparan sulfate-containing molecule, and the latter is an integrin. These studies should lead to the development of new therapeutic reagents.

The interaction of cells with extracellular matrix via integrin receptors induces signaling, cytoskeletal organization, growth, and protection from apoptosis. The newly described tumor suppressor PTEN/MMAC, which is mutated in 5-45% of a wide range of human tumors, was discovered by the Developmental Mechanisms Section to be a down-regulator of a variety of integrin-mediated functions, including cell migration, spreading, and signal transduction. Its specific protein targets are the key signaling proteins FAK (focal adhesion kinase) and Shc. Downstream, it affects MAP kinase signaling and two specific signal transduction pathways that regulate the velocity and directionality of cell migration. PTEN is also known to affect lipid signaling, and linkage to the protein signaling system was demonstrated. PTEN may be part of an important defense system against malignancy in which cells that leave behind normal extracellular matrix contacts undergo apoptosis (programmed cell death). Section members found that reconstitution of PTEN in three malignant cell lines missing this tumor suppressor restored regulation of FAK phosphorylation and apoptosis. The Cell Biology Section has identified novel extracellular regulators of cell migration and tumor metastasis. Ongoing studies on thymosin beta4 have established roles in endothelial, keratinocyte, and corneal epithelial cell migration. It accelerates in vivo wound repair in a rat skin biopsy model and in the cornea, suggesting its potential for promoting human wound healing. A novel function for osteonectin was identified as supporting prostate cancer cell metastasis to bone and induction of proteases. The cellular receptor has been identified as an integrin, and the amount of this integrin may serve as a diagnostic marker for metastatic breast and prostate cancer.

The molecular responses to extracellular matrix are being characterized in human salivary gland (HSG) cells and fibroblasts by the Cell Biology and Developmental Mechanisms Sections. When cells are placed on extracellular matrix proteins in cell culture, they show large changes in gene expression and protein biosynthesis. More than two dozen genes were identified as induced

by adhesion of salivary cells to collagen, fibronectin, or basement membrane extract, and many of them are novel. Salivary gland cells are also induced to differentiate and form mini-glands on basement membrane extract. This process involves synergism with growth factors, and the signaling mechanisms have been characterized. An 8-amino acid sequence in laminin is critical for this differentiation, and its cellular receptor has been found to be syndecan-1. These studies in cell culture systems are building the knowledge base necessary to develop creative therapeutic approaches for the repair or replacement of salivary glands and other tissues. For example, CDBRB is collaborating with the Gene Therapy and Therapeutics Branch to develop a first-generation artificial salivary gland. These and other exciting advances are described in the following list of publications and in reports on specific projects from each branch section.

Besides publishing extensively, our Branch distributes its research materials widely by licensing them, donating them to repositories, and providing numerous gifts to research colleagues. Products generated by CDBRB members that were licensed by companies included Matrigel and invasion substrates (Collaborative/Becton Dickinson and Sigma) and monoclonal antibodies against integrins (Becton Dickinson). CDBRB has donated hundreds of cDNA clones to the ATCC. We completed dozens of new, formal Material Transfer Agreements with extramural researchers this year to provide our reagents. Members of the branch have also received support from outside organizations. Significant support for research on proteoglycans came from Seikagaku. NASA provided funds to study salivary gland cell differentiation in microgravity. Non-NIH salary support for postdoctoral members of CDBRB has come from a wide variety of sources including the Japan Society for the Promotion of Science, the Dutch Cancer Society, the French CNRS, the German government, and the Spanish government.

CDBRB members continue to be invited as featured speakers at a variety of international meetings and symposia. Examples from this past fiscal year included H. Kleinman and K. Yamada at the 5th International Union of Biochemistry and Molecular Biology (IUBMB) Conference in Israel, Y. Yamada at the "International Conference on Molecular Interaction of Proteoglycans" in Japan, H. Kleinman at the Royal Society of Medicine Symposium on Angiogenesis in London, K. Yamada at the IADR symposium on "Dental and Craniofacial Research in the Post-Genome-Project Era" in Canada, and four CDBRB members at a variety of Gordon Conferences. Our members continue to serve on the editorial boards of a number of leading journals. Examples include J. Cell Biology (K. Yamada, Editor, and H. Kleinman, board member); Cancer Research (H. Kleinman, Associate Editor); J. Cellular Physiology (K. Yamada, Editor); Matrix Biology (Y. Yamada and K. Yamada, Associate Editors); J. National Cancer Institute (H. Kleinman, Associate Editor); and J. Cell Science (K. Yamada, board member). H. Kleinman also currently serves on four other journal boards, and K. Yamada serves on five others. Members also served on U.S. Army and NIH study sections (H. Kleinman and K. Yamada), on the Board of the Metastasis Research society (H. Kleinman), and on the Council of the International Society for Matrix Biology (K. Yamada). H. Kleinman was recently honored by the Bethesda AWIS Chapter Award for Mentoring. CDBRB members also provide extensive service on more than two dozen NIH and NIDCR committees, including the NIH Senior Biomedical Research Service Policy Board, NIH Diversity Council, NIH Scientific Conduct and Ethics Committee, and NIDCR Tenure and Promotion Committee

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Craniofacial Epidemiology and Genetics Branch

Scott Diehl
Albert Kingman
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CRANIOFACIAL EPIDEMIOLOGY AND GENETICS BRANCH
1999

The mission of the Craniofacial Epidemiology and Genetics Branch (CEGB) is focused on using epidemiological strategies and approaches to enhance understanding of the hereditary and environmental causes of dental, oral and craniofacial diseases and disorders. Such knowledge can be used to improve diagnosis, prevention and therapy. Nearly all work conducted in the Branch involves clinical research conducted on human subjects. Basic research involves the development and evaluation of new laboratory (genomic) assays and statistical methods designed to improve the power and efficiency of these research approaches, followed by application to ongoing studies being conducted in the Branch and elsewhere. Current studies of CEGB staff include large surveys of oral and systemic health based on interviews and examinations, case-control and cohort designs, and recruitment of nuclear and extended families. The focus of these investigations range from assessing health effects of amalgam, characterizing the oral manifestations of HIV and the oral physiology of aging, and improving understanding of the etiology of periodontitis and other causes of tooth loss, oral cancer, nasopharyngeal carcinoma, cleft lip and palate and other craniofacial disorders. Most studies are designed to evaluate both genetic variation and the behavioral and environmental risk factors associated with these diseases. Three Senior Investigators (S.I.s) are currently assigned to the CEGB of the Division of Intramural Research (DIR), NIDCR: Scott R. Diehl, Ph.D., Deborah M. Winn, Ph.D., and Albert Kingman, Ph.D. Most of the CEGB staff work at offices and computer facilities located in the Natcher Building on the main NIH campus. In addition, Dr. Diehl's gene mapping laboratory, located a short distance away at the National Naval Dental Center, is supported through an Interagency Agreement with the Navy.

Molecular Genetic Epidemiology

The mission of Dr. Diehl's research program is to increase our understanding of the etiology of dental, oral and craniofacial disorders by using the gene mapping strategies of association (disequilibrium) and linkage. Nearly all of his studies involve complex diseases, where multiple susceptibility genes are involved, and where gene-environment interactions are common. Although most studies utilize molecular assays such as marker polymorphisms or mutation analyses of candidate genes, several projects investigate familial aggregation of diseases and disease-related phenotypes without incorporation of molecular data. Analyses include key behavioral risk factors such as diet, smoking and alcohol consumption. These are treated both as covariates to disease risk and as genetically heritable phenotypes of interest themselves. The gene mapping strategy requires the following components, all of which have been successfully implemented by Dr. Diehl's research team: design and implementation of large scale field studies to obtain biospecimens and risk factor assessments for cases and appropriately matched controls; establishment of a laboratory capable of high-throughput genomic assays for both highly polymorphic DNA markers and candidate gene single nucleotide polymorphisms (SNPs); bioinformatics systems for management of biospecimens, molecular assay data, and clinical and risk factor information; and construction of computer hardware and software systems capable of conducting the thousands of statistical analyses required for genome wide studies by integrating a diverse array of computer programs in a user-friendly, semi-automated environment.

Dr. Diehl has collaborations with clinicians and epidemiologists throughout the world that have led to the successful establishment of several large patient collections for his research. Oral cancer cases and controls have been recruited in Taiwan and Greece, using both case-control and family-based sampling designs. A major gene mapping and risk factor study of nasopharyngeal carcinoma has been implemented in Taiwan, based primarily on multiplex families (families with 2 or more affected members). A very large study of syndromic and non-syndromic cleft lip and palate using both simplex and multiplex families has been completed at three clinical sites in the U.S. Studies of oral clefts in humans have been complemented by Quantitative Trait Locus (QTL) analyses of a mouse model of teratogen-induced clefting. A study of Kartagener syndrome has led to the mapping of a disease gene using families obtained through a collaboration in Poland. Kartagener syndrome is a form of primarily ciliary dyskinesia that has chronic sinusitis as a craniofacial manifestation. A whole genome scan and analyses of several candidate genes have been completed for a study of early onset periodontitis using families collected in the U.S., Chile, and Israel.

Completion of these major studies, several of which are currently in data collection phases, will be the focus of much of Dr. Diehl's research team's efforts during the next couple of years. Given the many study samples already in hand, it might appear that no additional clinical specimens need to be collected for years to come. However, improvements in genomic technologies can be expected to vastly increase the speed and scope of analyses that will be feasible for gene mapping laboratories. Dr. Diehl is committed to keeping his laboratory at the cutting edge of these new methods. Considering these likely technological advances in laboratory capabilities, ensuring future access to large numbers of biospecimens with high quality risk factor and demographic data will become an even more essential priority if this research program is to remain highly productive. Long term planning is especially important in this field, since it can take many years to establish collaborations, design and carry out large epidemiological studies. Based on this vision of the future, Dr. Diehl has recently entered into a long-term collaboration that will greatly expand his research program's access to oral cancer study samples at a worldwide level. He is also evaluating two new scientific opportunities for possible future studies. One involves genome scans and candidate gene studies of adult onset periodontitis. The adult form of this disease is much more common than early onset forms, and consequently may have a much greater impact on tooth loss in the post-fluoride era. A second new initiative involves joining with other DIR Senior Investigators to conduct human and animal model studies of the genetics of pain.

Analytical Epidemiology

The staff of the Analytical Epidemiology component, under the direction of Dr. Deborah Winn, has continued a program of scientific accomplishment and professional service. Their Intramural research includes studies of periodontal health and of oral and pharyngeal cancer. Early onset periodontitis (EOP) is a disease characterized by a progressive loss of the tooth supporting tissue in adolescents and young adults. In one study, a group of adolescents with early onset periodontitis and a control group of adolescents received an initial oral examination and a follow-up examination six years later. Advances have been made in understanding how levels of serum antibodies to P. gingivalis, P. intermedia, and A. actinomycetemcomitans relate to levels of these antibodies in gingival crevicular fluid, and how levels differ in control subjects and in

generalized and localized forms of EOP. Based on analysis of data from another study population, the Baltimore Longitudinal Study of Aging, pipe and cigar smoking appear to have the same adverse effects on periodontal disease as cigarette smoking. This finding is important in view of the recent surge in the popularity of cigar smoking. In another study, our analyses suggest that infection with P. gingivalis has a different pattern than for A. actinomycetemcomitans in the U.S. population, which may have implications for periodontal disease patterns in demographic and risk factor subgroups of the population.

Oral and pharyngeal cancers are characterized by a) alcohol- and tobacco-related etiology, b) low proportion of tumors identified at an early stage, c) racial differences in stage at presentation, d) racial disparities in survival even after controlling for stage, and e) approximately 50% survival rate. Several ongoing interrelated studies have as their goal the identification of risk factors for oral and pharyngeal cancer and identification of factors that influence detection of oral cancer or of persons at highest risk of these cancers. One project is the Puerto Rico Oral and Pharyngeal Cancer Study in which patients identified through a cancer registry are compared to the general population. The purpose is to identify genetic, behavioral, medical, and familial factors involved in the etiology of this disease. Recent key findings include evidence that oral and pharyngeal cancer risks decrease with cessation of tobacco and alcohol consumption, although risks of these cancers remains elevated for up to 20 years after cessation of use of these substances.

The SEER/Medicare Linkage Project is focused currently on a determination of patient medical care contacts occurring in the year prior to diagnosis of oral and pharyngeal cancer and the reasons for those contacts. Work to date suggests that physician visits are associated with earlier stage diagnosis for pharyngeal and laryngeal cancers, but not oral cancers. More seriously ill users of physician services are at greater risk for late-stage at diagnosis than those not as ill. These findings have implications for development of interventions for early detection of these cancers. In a related analysis, we have recently discovered a striking increase over time in the incidence of in-situ carcinomas of the oral cavity, pharynx and larynx.

NIDCR Chief Statistician

Dr. Albert Kingman has continued to serve in his capacity as NIDCR's Chief Statistician. His primary research interests include the following three areas: 1) design and analytical issues related to randomized clinical trials, including design issues in equivalence trials, with appropriate and efficient use of surrogate and multiple endpoints; 2) development of methodology in statistical genetics, focusing on linkage analysis and association studies; and 3) statistical issues in environmental risk assessment, especially related to exposure to dental amalgam and its potential health effects. Dr. Kingman is currently pursuing part-time sabbatical training in the field of genetic epidemiology at the Center for Inherited Disease Research in Baltimore. This center is a gene mapping facility jointly managed by the NIH and Johns Hopkins University. As part of this training experience, he is currently collaborating on several genetic epidemiology studies and working on new approaches to linkage analysis and association studies for human genome screens involving thousands of markers. He currently heads the Biostatistics Core, which provides training and consultation to the entire NIDCR on statistical design and analysis issues.

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Craniofacial and Skeletal Diseases Branch

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CRANIOFACIAL AND SKELETAL DISEASES BRANCH
1999

This Branch has focused its efforts on determining the processes by which skeletal elements are modeled during embryogenesis, remodeled and maintained in the post-natal organism, and how these processes are altered in disease states that affect the skeleton through the coordinated efforts of senior investigators working in the areas of developmental biology, cell and molecular biology, and protein and mineral chemistry. Furthermore, the translation of these efforts into clinical studies and applications has been, and remains a major area of emphasis in the Branch. The Branch has active collaborations with other NIH Institutes, institutions around the world, and participates in the Georgetown University Orthopaedic Residents Training Program. The Branch has made major advances in several areas, as highlighted below.

Studies in the Developmental Biology program performed by Dr. J. Terrig Thomas. This project is aimed at understanding the molecular basis of limb development and joint morphogenesis with special emphasis on signaling molecules. Cartilage-Derived Morphogenetic Proteins (CDMPs), polypeptides belong to the TGF-beta superfamily, and in situ hybridization studies have shown that CDMP-1 is the first gene to be found specifically expressed in presumptive joint interzones. With this in mind, we began to determine regulatory elements using transient transgenic experiments. However, due to the size of the promoter (100kb) it became difficult to identify specific elements. Consequently, we have taken advantage of the condensed genome of the puffer fish (*Fugu rubripes*). Cdmp-1 clones have been isolated from a puffer fish genomic library and the exon/intron structure is being characterized. In addition, this project has focused on the further characterization of the molecular basis of this Frzb inhibition of wnt gene signalling through a transgenic approach. The null phenotype appears to be lethal and consequently, we are generating a transgenic mouse line to express Cre recombinase specifically in facial mesenchyme under the control of the Msx-1 promoter, in order to delete Frzb in a conditional fashion. Mice are currently being tested for the tissue specificity of the Cre recombinase activity.

The Skeletal Biology program, directed by Dr. Pamela Gehron Robey, has focused on bone marrow stromal cells (BMSCs), which have the ability to form bone, cartilage, hematopoiesis-supportive stroma, associated fat cells, and perhaps other connective tissues as well, and are important mediators of skeletal metabolism in the post-natal organism. Previously, we had found that some, but not all, of the members of the stromal cell population maintain their ability to form bone, hematopoiesis supportive stroma and associated adipocytes. The differences between non-bone forming clones, clones that form bone were further investigated by examining their expression of receptor tyrosine kinases (RTKs), extracellular matrix proteins and transcription factors. Clonal populations of BMSCs were found to express varying levels of these RTKs, matrix proteins and transcription factors. It was found that there is a relatively high level of PDGF-R (beta) in bone-forming clones, and relatively high levels of EGF-R in non-bone forming clones, and that the rate of proliferation of bone-forming clones is positively correlated with the amount of bone formed in vivo. In collaboration with investigators at the University of North Carolina, we have used a new approach to isolate and characterize human cementoblasts for the first time. These cells were found to be similar, but not identical to cells that produce bone. This provides an excellent model system to study the physiology of this unusual cell type,

and for future design of periodontal reconstructions. The program has continued its collaboration with members of NHGRI in the Skeletal Genome Anatomy Project (SGAP) which is designed to aid in gene discovery and to determine changes in the pattern of gene expression of skeletally derived cells as a function of developmental age and of disease processes. To date, over 8,000 clones from two of our libraries have been sequenced and are being analyzed for novel and unknown gene expression, and the pattern of gene expression within two different libraries is being compared.

The matrix proteins of bones and teeth play key roles in the structure and functions of these tissues. The objective of the Molecular Biology of Bones and Teeth program, lead by Dr. Marian F. Young, was to study their function and regulation using a combination of in vitro and in vivo analysis. A major challenge for the study of human gene function and regulation is the extreme inefficiency of DNA transfer DNA into human non-transformed cultured cells. In order to devise new methods to overcome this blockade we tested two adenoviral-based procedures using human bone marrow stromal cells and trabecular bone cells. Adenoviruses containing beta-galactosidase recombinant genes showed substantial gene transfer indicating they are ideal for these unique cell types. We also tested adenoviruses modified with poly-lysine additionally and found they transferred genes into multilayer highly differentiated non-dividing cells also with high efficiency. We used this methodology to "rescue" estrogen responsiveness in cells unable to make the estrogen receptor alpha (HERKO). Our data showed that ERalpha dependent gene activation by estrogen was possible and, further, was influenced by treatment with TGF-beta1. IL6 production in ER rescued HERKO was also influenced by treatment with TGF-beta. This is the first approach of its kind to clarify the relationship of growth factors to the function of the estrogen receptor human bone. To determine the function of matrix proteins in vivo we generated bgn-deficient mice. While apparently normal at birth, these mice display a phenotype characterized by reduced growth and bone mass due to the absence of *bgn*. Subsequent analysis has shown that there is also an alteration in biomineralization in these mice, which most likely contributes to their osteoporosis-like phenotype. These mice may serve as an animal model to study the role of ECM proteins in osteoporosis.

The Matrix Biochemistry program, headed by Dr. Larry Fisher, has continued its pursuit of the structure-function relationship of the major non-collagenous proteins of bones and teeth. With our collaborators in Germany we have continued to study the endocytosis receptor for the two small proteoglycans, biglycan and decorin. These same molecules have also been shown to support normal endothelial development in vitro. We are also working closely with Dr. Young's group in understanding the skeletal phenotype of the biglycan and decorin knockout mice. Another major effort of the group has been to continue the study of the family of integrin-binding glycoproteins, bone sialoprotein (BSP), osteopontin (OPN) and dentin matrix protein 1 (DMP1). With our collaborators in Belgium we have extended our previous observation that BSP is a marker of osteotropic cancers to prostate cancer. Indeed, higher levels of BSP expression in prostate tumors was shown to correlate to a three-fold increased likelihood of relapse when compared to patients with tumors expressing no or low levels of BSP. We have filed a preliminary patent application on our concept that the levels of BSP serum and/or urine may be a marker of various cancers as well as certain bone diseases. Furthermore, we have developed a new assay that promotes the selective amplification of a mutant allele during polymerase chain reaction (PCR) even if the allele is present in only a small fraction of the cells.

The amplicon is then sequenced to determine the specific mutation in the tissue sample or cell culture. While we have currently limited our use of this technology to somatic mutations that cause MAS and fibrous dysplasia of the skeleton, this approach should be equally useful for cancer studies or any other disease in which post-zygotic mutations contribute to human disease.

The research of the Mineral Chemistry and Structure program, headed by E. D. Eanes, continued to focus on biologically relevant calcium phosphate salts. A major effort was directed toward delineating the role of the solution state, especially anionic electrolytes, in establishing the size and shape of crystals of apatite, the dominant mineral phase in skeletal tissues. Findings revealed that the effects of anionic electrolytes on crystal size were dependent on how strongly they inhibited growth kinetics. Weak inhibitors such as serum albumin and citrate limited the size that the crystals could attain by suppressing growth in width and thickness. In contrast, stronger inhibitors such as bisphosphonates, acidic polypeptides, and phosvitin stimulated growth in these lateral dimensions. None of the inhibitors studied affected growth in crystal length. These in vitro findings suggest that polyanions in the extracellular fluid of skeletal tissues may have a significant collateral role in controlling the size and shape of apatite crystals in these tissues. Previous work demonstrated that amorphous calcium phosphate (ACP)-methacrylate composites could possibly find uses as mineralizing agents in clinical dentistry. Current studies showed that the mechanical strength and the calcium and phosphate ion releasing properties of these materials could be improved by modifying the ACP filler with glass-forming agents such as tetraethoxysilane and zirconyl chloride. Such improvements should extend the range of application of these composites as dental materials.

The Skeletal Clinical Studies program, currently under the direction of Dr. Pamela Gehron Robey, has established three clinical protocols (98-D-0145, 98-D-0146, 99-D-003) and participates in a fourth (97-DK-0057) for the study and treatment of fibrous dysplasia of bone (FD) and the McCune-Albright Syndrome (MAS). FD is found in a broad range of severities, ranging from monostotic (single bone) to polyostotic (many bones) and often in association with the MAS, which in addition to FD has multiple endocrinopathies and skin hyperpigmentation. MAS is known to arise from a post-zygotic mutation in the GNAS1 gene (R201C and R201H). An in-depth histological and molecular study of FD lesions revealed a great deal of variation in the nature of the lesions, and aided by the development of a novel assay by Dr. Larry Fisher, all of the patients were found to have GNAS1 mutated cells. In addition, we also identified a novel R201G mutation in one of our patients. These results indicate that GNAS1 mutations result in a broad spectrum of bone lesions even outside of the context of MAS, and that new therapies may have a broader impact than previously realized. Although it has been known since the 1960's that bone marrow stroma contains a population of cells that have the ability to form bone, cartilage, myleosupportive stroma, adipocytes and perhaps other connective tissues, it is only recently their utilization for bone regeneration has been fully realized, and the program has developed procedures whereby ex vivo expanded bone marrow stromal cells could be used in tissue regeneration. Defects were created in the skulls of mice that were of sufficient size that they would never spontaneously heal. When these defects were filled with a composite of ex vivo expanded bone marrow stromal cells and an appropriate carrier, bone and marrow were completely regenerated. These studies have served as the basis for development of procedures for use in humans with similar, non-healing bone defects. An application has been submitted to the FDA, and it is hoped that clinical trials will begin in the very near future.

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Gene Therapy and Therapeutics Branch

Bruce Baum
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GENE THERAPY AND THERAPEUTICS BRANCH
1999

The Gene Therapy and Therapeutics Branch (GTTB) provides a bench to clinic continuum focusing on questions related to salivary gland biology and pathology. This reporting period has seen not only substantial scientific progress, but also two significant changes in our senior staff. Philip Fox and Brian O'Connell have departed the NIH to assume senior positions elsewhere (Director, R & D at Amarillo Biosciences; and Professor, Trinity College-Dublin, respectively). After a national search, we recently selected a new senior staff member, John Chiorini, who will join GTTB and establish a program in adenoassociated virus biology. GTTB remains committed to the notion that significant advances in clinical care will come from our understanding of biological mechanisms and our inter-disciplinary approach.

The production of salivary fluid is due to the transepithelial secretion of Cl^- by acinar cells. A Na^+-K^+-$2Cl^-$ cotransporter located in the acinar basolateral membrane drives much of the Cl^- secretion. This transporter is the rate-limiting step in salivary secretion. The Membrane Biology Section (MBS) has concentrated on understanding the function and regulation of this important transport protein. The MBS demonstrated that up-regulation of this cotransporter occurs in rat parotid acini in response to several physiologically related stimuli. For example, β-adrenergic stimulation led to phosphorylation of the cotransport protein itself, with there being a close correlation between increased Na^+-K^+-$2Cl^-$ cotransporter activity and increased cotransporter phosphorylation. These effects are paralleled by an increase in the number of high affinity binding sites for the cotransporter inhibitor bumetanide in acinar membranes. Since the sensitivity of cotransporter fluxes to inhibition by bumetanide is the same in both resting and isoproterenol-stimulated cells, β-adrenergic stimulation likely results in the activation of previously quiescent transporters rather than in a change in the properties of already active proteins.

Neurotransmitter stimulation of sustained salivary fluid secretion depends on the activation of store-operated Ca^{2+} influx. The molecular mechanism involved in this process is unknown for all non-excitable cell types. GTTB's Secretory Physiology Section (SPS) has directed much effort towards identifying the component(s) mediating Ca^{2+} influx using salivary gland cells as models. SPS scientists focused on components of the transient receptor potential *(trp)* gene family as possibly encoding the store-operated Ca^{2+} influx channel (SOC). They demonstrated for the first time that the localization of the Trp1 protein in an intact non-excitable tissue, rat submandibular gland, is consistent with its proposed function in the plasma membrane. Further, they showed that HSG cells (a human submandibular line) stably transfected with human (h) *trp1* cDNA displays (i) a higher level of hTrp1 protein and (ii) a 2-3 fold increase in SOC activity. Further, transfection of HSG cells with antisense h*trp1* cDNA significantly attenuates stimulation of SOC activity. These data provide strong evidence for the involvement of the Trp1 protein in the SOC mechanism in salivary gland cells. Additional work by the SPS showed that the Trp1 protein interacts with proteins in a lipid raft domain likely via its ankyrin- and dystrophin-like domains. Manipulations that disrupt these domains in HSG cells lead to an attenuation in muscarinic-stimulated Ca^{2+} signaling and SOC activity.

A second major effort by the SPS involves their finding that a renal outer medullary K^+ (ROMK)-type channel is present in HSG cells and is specifically activated by high [ATP]. Low [ATP] activates internal Ca^{2+} release, an outward Ca^{2+}-dependent K^+ channel, and an inward store-operated Ca^{2+} current (I_{SOC}), while high [ATP] activates an inwardly rectifying K^+ current, without increasing $[Ca^{2+}]_i$. These and other data demonstrate for the first time that a ROMK-type $K_{(ATP)}$ channel is present in salivary gland duct cells. Further, it suggests a novel mechanism for K^+ secretion by these cells into the forming saliva.

The Gene Transfer Section (GTS) studies clinically relevant applications of gene transfer to salivary glands. Much of this work has employed replication-deficient recombinant adenoviral vectors. These vectors are useful for establishing proofs of clinical or biological principle. However, adenoviral-mediated transgene expression is transient and vectors elicit a potent immune response. The GTS continued its efforts to minimize this vector-elicited immune response with two studies in murine salivary glands. The first used a cationic liposome-encapsulated bisphosphonate, clodronate, that can deplete the liver of phagocytic cells. However, clodronate delivery to submandibular glands, by intravenous, intrarterial, intraperitoneal, or intraductal routes, was unable either to deplete glands of macrophages or increase transgene expression. Secondly, GTS scientists administered an adenoviral vector encoding an anti-inflammatory cytokine (either IL-4 or IL-10) along with an adenovirus encoding a reporter gene. The co-administration of the IL-10 vector, but not the IL-4 vector, led to a marked reduction in chronic inflammatory cells in submandibular glands 28 days after vector administration, but without a significant increase in reporter gene expression.

In addition to trying to manipulate such immune responses, the GTS increased efforts to develop alternative means of transferring genes. One approach employs recombinant adenoassociated virus (rAAV) vectors. The GTS previously demonstrated that rAAVs can transduce murine salivary epithelial cells in vivo. Those studies utilized the classical method of generating rAAVs that includes infection by a helper adenovirus and leads to rAAV contamination by adenoviral proteins. During this reporting period we have replaced the helper virus with a plasmid encoding necessary adenoviral genes. The resulting rAAV includes the relatively salivary tissue-specific kallikrein promoter, is produced at slightly higher (~3-fold) titers than earlier rAAVs, and is able to transduce murine submandibular gland epithelial cells in vivo. Another gene transfer approach employs cationic liposomes generated with GAP-DLRIE/DOPE. Lipid-mediated human growth hormone (hGH) expression, however, was 0.16-0.35% of that resulting from adenoviral (10^8 pfu)-mediated hGH expression. A third approach involved the development of a hybrid vector including both adenoviral and retroviral (Moloney murine leukemia virus; MoMLV) elements. These two virus types individually possess useful characteristics for gene therapy. Adenoviruses can be produced at high titers and can infect many cell types. Retroviruses can integrate into the host cell chromosome and lead to stable transgene expression. The resulting hybrid vector carries the 5' and 3' long terminal repeat sequences from MoMLV, and a luciferase reporter gene. This vector was able to infect dividing and non- dividing cells in vitro, as well as submandibular glands and brain in vivo. Four different experimental approaches (PCR, Southern hybridization, Fluorescence In Situ Hybridization, and gene walking) consistently showed that the reporter gene was integrated into the host cell chromosome. This novel vector may have broad applicability for human gene therapy.

The GTS previously showed that salivary glands are able to secrete transgene-encoded proteins into serum as well as saliva. Recently, the GTS assessed if transgene-encoded therapeutic proteins can follow distinct, polarized sorting pathways in vivo as have been shown to occur classically in cell biological studies in vitro. Four, first generation, E1$^-$, type 5 recombinant adenoviruses were used to deliver different transgenes to rat submandibular glands. The results are consistent with the recognition of signals encoded within the transgenes that lead to specific patterns of polarized protein secretion. The eventual understanding of these signals, and their manipulation, should allow for efficient delivery of therapeutic proteins from salivary glands to specific sites of function.

Patients with severe salivary hypofunction (irradiated; Sjogren's syndrome [SS]) often lack parenchymal cells and are not candidates for gene therapy approaches. The GTS is trying to develop an "artificial salivary gland" to implant intraorally. This device requires three components; (i) a biodegradable substratum, (ii) a luminal coating of an extracellular matrix protein(s), and (iii) a polarized, water permeable monolayer of epithelial cells capable of generating an osmotic gradient (lumen>interstitium). We examined two model biodegradable substrates; poly-(L)-lactic acid (PLLA) and polyglycolic acid, four individual matrix proteins (fibronectin [FN], laminin, collagen I, and collagen IV), and a potential allogeneic graft cell line, HSG. Optimal results were obtained with cells grown on PLLA to which FN was pre-absorbed. HSG cells were able to form an epithelioid monolayer, dependent on time and FN concentration.

GTTB clinical studies have been restructured over the past year to improve how studies are designed, reviewed and managed. The principal investigative goal of our SS Clinic is to determine if salivary dysfunction can be ameliorated by immunomodulatory therapy. There are few rigorous trials to develop treatments for SS, despite there being an estimated 1 million persons in the USA with this disorder. Over the past year, more than 400 patients were seen in the SS Clinic. Accrual in the randomized, placebo-controlled trial of dehydroepiandrosterone is almost complete. Accrual has just begun for a similar study examining the efficacy of thalidomide treatment for SS management. This year we also began a hypothesis-driven natural history study. This addresses: (i) the long term effects of SS on oral and systemic health; (ii) the development and progression of B-cell dysregulation in SS; (iii) whether diagnostically incomplete SS will progress to diagnostically complete SS; and (iv) the development of rigorous outcome measures for following patients with this disorder.

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Oral Infection and Immunity Branch

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ORAL INFECTION AND IMMUNITY BRANCH
1999

The Oral Infection and Immunity Branch plans, fosters and carries out research relating to the causes, diagnosis, treatment and prevention of infectious and inflammatory diseases. Efforts to understand the functional and molecular organization of infectious organisms, and research into the cellular, biochemical and molecular components of inflammatory and immune responses provide the basis for dissecting the interactions between pathogens and the host immune system. These multifaceted approaches define fundamental mechanisms of host defense, how these pathways become dysregulated to become pathogenic, and how to intervene for the benefit of the host.

The programs within the Oral Infection and Immunity Branch range from basic to translational research, and now encompass clinical activities, including a Bench-to-Bedside Award from the NIH Office of the Director and Clinical Center. Furthermore, as part of our commitment to the pursuit of clinical research, we have established formal training programs with Children's National Medical Center for infectious disease Fellows and with the Department of Periodontology at the University of Maryland for periodontal disease Fellows. In addition to mentoring, Senior Investigators in the Branch continue to receive local, national and international recognition for their successful research programs and service-oriented activities. Recognition in the form of external funding comes from DARPA and NCI for exploration of mechanisms of anthrax relevant to bioterrorism, from NIDDK for diabetes research, and from Targeted Genetics, Inc. for novel approaches to gene therapy. Invitations to organize, chair, and speak at international meetings, membership on editorial boards, invitations to edit books and write authoritative chapters, and election as officers in scientific societies all attest to the high caliber of our staff and the respect afforded to them. Within the Branch, OIIB Employee of the Quarter Awards also recognized special achievements.

In addition to a major focus this year on our Board of Scientific Counselors Review, and initiation of phase one of a multi-year renovation project, investigators in the Branch have had a stellar year. Important new findings and scientific breakthroughs, including those featured on the covers of prestigious journals such as *Cell* and *Journal of Experimental Medicine,* are highlighted in this Annual Report.

A collaborative effort between scientists in OIIB and UCSD identified the genes responsible for the mammalian sense of taste. In *Cell*, these investigators described genes that encode two novel proteins, TR1 and TR2, uniquely expressed in cells within taste buds of the tongue and palate epithelium which are responsible for the sense of taste. The isolation of the candidate taste receptor genes, members of a group of G protein-coupled receptors, provides the groundwork necessary for manipulating the perception of taste and devising methods to stimulate or block taste cell function, as well as deciphering how the sense of taste is connected from the oral cavity to the brain.

The oral cavity also serves as an entry point for numerous infectious organisms. Viridans streptococci including *S. gordonii* are the predominant primary colonizers of the tooth surface. Specific functional properties of bacterial cell surface structures, particularly the receptor

polysaccharides are required for oral microbial adhesion, colonization and subsequent initiation of inflammation. Recent identification of six structurally-related streptococcal receptor polysaccharides uncovered the presence of a host-like motif in the repeating unit of each polysaccharide which accounts for their recognition by the adhesins of other oral bacteria. Importantly, the host-like features of these receptor polysaccharides appear relatively non-immunogenic, suggesting a possible mechanism for evasion of the host immune response. Molecular probing of a *S. gordonii* 38 genomic DNA library identified a new operon composed of four genes which direct biosynthesis of polysaccharide precursors. Further studies utilizing differential PCR revealed that a DNA product which encodes the saliva-binding surface adhesion proteins, SspA and SspB, was induced in *S. gordonii* by exposure to saliva and facilitates adhesion. Since most oral viridans streptococci, in addition to intergeneric coaggregation with actinomyces, participate in intrageneric coaggregations, a gene identified by transposon mutagenesis and shown to be homologous to *dlt*A of *L. rhamnosus* may provide new insight into such interactions. DltA encodes a ligase involved in incorporation of D-alanine into lipoteichoic acid, a potential adhesin binding site. In addition to streptococcal coaggregation, fusobacteria, which are the most numerous gram-negative plaque organisms, act as bridge organisms by coaggregating with both early and late colonizers. Molecular characterization of these events indicates that the *mal*-operon in *F. mortiferum* and *B. subtilis* is comprised of three genes encoding a sugar-specific transport protein, a phospho-alpha-glucosidase, and a polypeptide of unknown function, designated *yfi*A, which may serve as a transcriptional regulator for expression of the *mal*-operon and a regulatory target.

Understanding the adhesive and virulence factors of pathogens not only provides targets for vaccines and other means to inhibit infection, but may also provide an approach to redirect the virulence factors or toxins for the benefit of the host. For example, anthrax toxin protective antigen (PA) binds to cell surface receptors, is cleaved by the cell surface protease furin, and then captures either lethal factor or edema factor for delivery to the cytosol to kill the target cells. To achieve cell-type specific delivery of toxin-fusion proteins, PA has been cleverly modified by replacing the furin site with sequences which are cleaved by matrix metalloproteases (MMP). Two PA-MMP fusion proteins were produced and shown to be cleaved in MMP-synthesizing tumor cells. Since activation of the PA mutant proteins occurs only on the cell surface, cell and tissue specificity may be retained *in vivo*. Such increased understanding of the pathogenic mechanisms associated with microbial infections may also identify novel vaccine and therapeutic strategies to protect humans from the effects of infectious diseases. In clinical isolates of *S. pneumoniae*, a specific single amino acid mutation in the bacterial chromosomal gene for dihydrofolate reductase was shown to be responsible for trimethoprim resistance and a structural model was developed to facilitate the design of new antibacterial agents against S. *pneumoniae*. Moreover, resistance to optochin was also identified in S. *pneumoniae* and traced to single amino acid mutations in the ATPase encoding genes.

Infection and injury elicit a complex series of reactions in the host designed to isolate and/or eliminate the inciting agent(s) as well as to minimize and repair tissue damage. Precise regulation of these mechanisms is crucial for the maintenance of tissue integrity, and malfunction may result in pathologic responses and/or tissue destruction. Mast cells play an important role in such reactions by releasing an array of mediators and efforts continue to focus on understanding the underlying intracellular signal transduction pathways. Protein tyrosine phosphorylation of

the tyrosine kinase Syk is an early and critical signal for Fc epsilon RI-induced mast cell degranulation. Mutational analyses revealed that Syk activation loop tyrosines are necessary for Syk mediated propagation of signaling. A putative Syk phosphorylation site in the linker region of Syk functions to negatively regulate its kinase activity and thereby disrupt the signals leading to the release of inflammatory mediators. New roles have also been revealed for the protein tyrosine phosphatases SHP-1 and SHP-2 in regulating mast cell cytokine production.

What actually triggers aberrant immune responses in autoimmune diseases, including insulin-dependent diabetes mellitus (IDDM), remains unclear. Although it has been speculated that some IA-2 autoreactive T-cells may escape negative selection and initiate an autoimmune response in the presence of high risk HLA alleles, OIIB investigators examined the possibility that the amino acid sequence of the major autoantigen IA-2 may be different in subjects who go on to develop an autoimmune disease. Using genomic DNA, they have shown that IA-2 and IA-2beta, both of which are major autoantigens in IDDM, are members of the protein tyrosine phosphatase (PTP) family, but because of two amino acid substitutions lack enzyme activity. In an extension of these studies, homologs of IA-2 and IA-2beta have been uncovered in *C. elegans* and Drosophila placing these molecules in a new subgroup of the PTP superfamily. An extremely important outcome from these studies is that autoantibodies to IA-2 and IA-2beta, as well as to glutamic acid decarboxylase, appear years before the development of clinically apparent IDDM and therefore, can serve as predictive markers for this disease. Screening for double-positive individuals is now being widely developed to study the pathogenesis of IDDM and to select for therapeutic intervention trials.

In another autoimmune disease, degenerative inflammatory arthritis, degradation of the proteoglycans and collagen of articular cartilage has been shown to be mediated by excess neutral serine proteases and metalloproteinases. A prominent cell type in effusions of inflamed joints is the neutrophil, the source of two serine proteases, elastase and cathepsin G. Disruption of the balance between proteases and protease inhibitors is often associated with pathologic tissue destruction. To explore the therapeutic potential of secretory leukocyte protease inhibitor (SLPI), a serine protease inhibitor, in erosive joint diseases, rat SLPI was cloned, sequenced, expressed and injected into rats with bacterial cell wall induced erosive polyarthritis. SLPI inhibited joint inflammation and cartilage and bone destruction as detected by circulating levels of type II collagen collagenase-generated cleavage products. TNF and NF kappa B activation were also diminished by SLPI, indicating that the action of SLPI may extend beyond inhibition of serine proteases.

Acute and chronic wound healing morbidity in the elderly imposes a substantial burden on health services since delays in cutaneous wound healing can lead to local infection, wound dehiscence, and a shift towards chronic wound repair. New studies have shown that topical or systemic estrogen can reverse age-related changes in acute wound healing. This acceleration of healing was associated with a reduction in neutrophils, elastase and tissue breakdown, but increased monocytes and TGF-beta. *In vitro* mechanistic studies suggest that estrogen and TGF-beta have direct effects on inflammatory cell-derived cytokines and cell adhesion molecules. Moreover, *in vivo* animal studies suggest that specific TGF-beta signaling pathways involving Smad3 are pivotal in recruiting inflammatory cells to the wound, and disruption of these pathways results in altered wound healing kinetics. Compelling evidence also reveals that the elastase inhibitor,

SLPI, interrupts the proteolytic cascade to facilitate the wound healing process. Genetic deletion of SLPI results in a marked delay in healing, and represents the basis of a 'Bench-to-Bedside' proposal to assess the effects of SLPI on acute standardized wounds.

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Oral and Pharyngeal Cancer Branch

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ORAL AND PHARYNGEAL CANCER BRANCH

1999

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious health problem based on annual morbidity and mortality rates. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still largely unknown. Members of our Branch work on several complementary aspects of cancer cell biology, in an effort to understand the molecular basis for malignant transformation as well as to use this knowledge to develop molecular markers of disease progression and novel therapeutic approaches for oral malignancies.

During the current reporting period, we have strategically initiated programs and recruited staff in areas which are relevant to oral cancer research. This includes the appointment of Dr. Thomas Bugge, Principal Investigator, to head a new unit focused on the role of the large family of tissue proteases in wound healing, tissue remodeling and regeneration, and metastasis, and the expansion of Dr. Myung Hee Park, Dr. Frank Robey, and Dr. Gutkind's research programs in the areas of oral epithelial cell proliferation, differentiation, and neoplastic transformation. This new direction is now affording us a better understanding of normal epithelial cell biology, which will likely broaden the horizon on developing potential oral tumor markers and treatments. Furthermore, our Branch continues to play a leading role in a newly formed NCI/NIDCR/NIDCD Inter-Institute Consortium in Head and Neck Cancer, whose goal is the development of a comprehensive clinical research program in head and neck cancer at the NIH, based on the interdisciplinary expertise of the intra- and extramural components of the three institutes.

Our Branch has been highly productive during this year, and has made substantial contributions to the field, providing new concepts and shedding new light on questions of fundamental importance for cancer biology. We have also developed a large number of novel reagents, such as new genes, expression vectors, cell lines, antibodies, and bioactive peptides of value to biomedical research, and provided them to hundreds of investigators in the U.S. and abroad. Another tradition of our Branch has been our high priority on the training of postdoctoral investigators to become independent leaders in the field; we have tried to continue and strengthen this commitment.

During the current reporting period, progress has been made in a number of research efforts at the OPCB. A variety of arbitrarily selected research advances are highlighted below. The progress report for each project provides a more comprehensive description of the major findings in our Branch.

We have recently established the Head and Neck Cancer Genome Anatomy Project (HN-CGAP), a cooperative effort with the NCI's CGAP. A number of cDNA libraries from HNSCC cell lines and normal and immortalized gingival keratinocytes have been submitted and partially sequenced. Currently available information already suggests a distinct pattern of gene expression in these cells. Furthermore, this work helped identify 39 previously unknown cDNA clones. Recently, several high quality cDNA libraries from microdissected normal and neoplastic tissues from the oral cavity have been engineered, and preliminary sequence analysis has already

identified more than one hundred potentially novel genes. Furthermore, the pattern of expression of novel and known genes is now being evaluated by DNA-chip screening methodologies. This work is expected to help identify gene products involved in the neoplastic process, as well as novel molecules representing clinically useful markers of oral carcinogenesis.

We have continued our drug evaluation effort at the NIDCR, whose goal is to develop novel therapies aimed at improving the quality of life and life expectancy of oral cancer patients. As part of this project, we have recently evaluated newly identified drug candidates for their effectiveness in squamous cell carcinomas as a collaborative effort with the Developmental Therapeutic Program, NCI. One such drug candidate, flavopiridol, potently inhibited the proliferation of human squamous carcinoma cells and dramatically reduces tumor growth *in vivo*, thus establishing flavopiridol as a suitable drug candidate for treatment of head and neck carcinomas. Based on these encouraging results, a Phase 2 trial with flavopiridol for HNSCC is planned to be conducted shortly, as part as a joint effort with NCI and NIDCD. For the first time, the evaluation of molecular end points, based on the use of laser capture microdissection and gene arrays, will be also an integral component of these clinical studies. In preparation, extensive analysis of the changes in gene expression provoked by flavopiridol and other chemotherapeutic agents and gamma radiation in HNSCC cell lines is being conducted, in collaboration with DCS, NCI.

The Kaposi's sarcoma associated herpesvirus (KSHV/ HHV 8) is implicated in the pathogenesis acquired immunodeficiency syndrome-associated Kaposi's sarcoma (AIDS-KS), the most common malignancy in human HIV infection. The oral cavity is frequently involved in AIDS-KS and may represent one of the most frequent initial sites of this malignancy. As part of a collaborative effort, we have recently shown that a G protein-coupled receptor (GPCR) encoded by the ORF 74 of KSHV (KSHV-GPCR) displays constitutive activity, and is able to stimulate cell proliferation and expression of angiogenic growth factors, such as VEGF. We have recently found that the KSHV-GPCR enhances the expression of VEGF by stimulating the activity of the transcription factor HIF-1 alpha, which binds to and activates transcription from a hypoxia response element within the VEGF promoter. This stimulation involves the direct phosphorylation of HIF-1 alpha by MAPK. These findings provide the first insight into a mechanism whereby growth factors and oncogenes acting upstream from MAPK can interact with the hypoxia-dependent machinery of angiogenesis to stimulate VEGF expression.

Receptors coupled to heterotrimeric G proteins (GPCRs) can effectively stimulate growth promoting pathways in a large variety of cell types, and if persistently activated, these receptors can also behave as dominant-acting oncoproteins. We have investigated the nature of the mitogenic and transforming pathways elicited by GPCRs as an experimental system for uncovering novel biochemical routes participating in the transduction of proliferative signals. In this reporting period, we have focused on how this family of cell surface receptors induces the expression of the c-*jun* proto-oncogene. We have now found that GPCRs can activate the c-*jun* promoter in a JNK-independent manner. Instead, we have demonstrated that GPCRs can elevate the activity of novel members of the MAP kinase family, including ERK5, p38 alpha, p38 gamma and p38 delta and that the activation of certain kinases acting downstream from MEK5 (ERK5) and MKK6 (p38 alpha and p38 gamma) is necessary to fully activate the c-*jun* promoter. Taken together, these results suggest that the pathway linking GPCRs to the c-*jun* promoter involves the integration of numerous signals transduced by a highly complex network of MAP

kinases, rather than resulting from the stimulation of a single linear protein kinase cascade. We have investigated the growth and differentiation properties of normal human gingival keratinocytes (NHGK) and found that the [Ca^{++}] optimum for culture and the squamous envelope composition of oral keratinocytes are different from those of normal human skin keratinocytes. We have also examined known markers of differentiation and found major differences in the expression of transglutaminase (TGase) I, TGase II and cytokeratins between NHGK and several head and neck cancer cell lines. For example, the level of TGase II activity of the HN cells appeared to correlate with their ability to grow in soft agar, suggesting a role for TGase II in anchorage-independent growth. In order to evaluate the roles of various proto-oncogenes and activated oncogenes in oral carcinogenesis, we introduced various oncogenes into immortalized human gingival keratinocytes (IHGK). Immortalization of NHGK was accomplished by transfection with the pLXSN vector or the pBabe-Hygro vector carrying HPV16 E6/E7 genes, and immortalized cell lines were established after continued passage (>50) in vitro. IHGK express a similar pattern of cytokeratins as do NHGK and appear to retain the ability to induce TGase I and terminal differentiation after reaching post-confluence in media containing Ca^{++} at 0.15 mM. Various oncogenes implicated in head and neck squamous cell carcinomas (SCC), including N-ras, K-ras, c-Raf, c-myc, and as a control the viral oncogene MSV-H-ras and its activated form, MSV-H-ras-V12, were individually transfected into IHGK. Biological and biochemical properties of these newly established cell lines are under current investigation.

Patients suffering from head and neck squamous cell carcinomas may have significant deficiencies in natural cellular and humoral immunity. Particularly, severe cellular immunosuppression can be detected early in the disease, and the cause of this is not clear yet. We have been building on our knowledge of immunosuppression caused by the envelope protein from HIV, gp120, as a template for studying HNSCC-induced immunosuppression. Since IL-2 is considered to be critical for the immune system to function normally, efforts during this reporting period have addressed novel mechanisms interfering with IL-2 production. For several years, our work has been focused on the C4 domain of gp120. The C4 domain in gp120 is the major region in gp120 that binds to CD4. This year we were able to successfully design and make a cyclized form of the C4 peptide that competed with gp120 for binding to CD4 and, most importantly, we were able to use this cyclic C4 as an immunogen to produce rabbit anti C4 antibodies that react with gp120 and block the binding of gp120 to CD4. A polymerized form of the C4 peptide was able to cause the attenuation of IL-2 and IL-2R expression in T cells. Understanding the molecular mechanisms of immunosuppression caused by HIV should lead to an understanding of cancer-induced immunosuppression.

We have utilized keratinocytes of murine epidermis as a well-characterized biological model of squamous morphogenesis, and both *in vivo* and *in vitro* approaches to dissect the stages of differentiation and neoplastic progression. Our major focus has been on the role of the tumor suppressor p53, downstream effectors, and related proteins. We have shown that loss of the p53 mediated gene *WAF1* enhances papilloma formation but has no effect on malignant conversion, suggesting that different p53 mediated functions contribute to distinct stages of the neoplastic process. In addition, we are characterizing the function and mechanism of action of a novel mitochondrial chloride channel protein, the expression of which is regulated by p53 and TNF-alpha. Over-expression of this protein induces apoptosis in several cell types including keratinocytes. Additional efforts are directed towards developing animal models to target genes

to the oral epithelium, and exploring the role of specific signal transduction molecules, including some that are aberrantly expressed in head and neck cancers, in a keratinocyte model.

ORAL AND PHARYNGEAL CANCER BRANCH
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Pain and Neurosensory Mechanisms Branch

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PAIN AND NEUROSENSORY MECHANISMS BRANCH
1999

The Pain and Neurosensory Mechanisms Branch (PNMB) conducts a multidisciplinary research program aimed at improved understanding and treatment of pain. Studies range from evaluating molecular responses to tissue injury and elucidating the mechanisms of peripheral tissue inflammation, including subsequent changes within the nervous system, to evaluating novel drugs and clinical hypotheses about pain and its control in human models of acute and chronic pain. The hallmark of the Branch's research program is the integration of basic and clinical research which permits not only a rapid transfer of new findings from the laboratory to the clinic, but also fosters basic research based on clinical problems. This integrative approach provides an optimal environment for training clinicians and basic researchers in the principles and methods of pain research across a spectrum spanning basic molecular mechanisms to the clinical management of pain. In addition, the Branch's senior investigators participate widely in speaking, writing, and collaboration with professional organizations, academic institutions, and patient advocacy groups to transfer emerging scientific information to the training of clinicians and the treatment of patients.

The independence and challenge presented by the NIDCR reorganization and the opportunities for PI-initiated research have increased scientific vigor and productivity in the Branch. The Branch continues to operate a large clinical research program, the Pain Research Clinic, in the Magnuson Ambulatory Clinical Research Facility under the scientific direction of Drs. Max, Dionne and Gracely. Research conducted in this clinic is often based on observations made in the Branch's basic laboratories as well as using novel and prototypic drugs to test emerging scientific hypotheses in man, representing a true 'molecules to man' continuum. The research activities of the Branch were prominently featured at the 1999 meeting of the International Association for the Study of Pain: Dr. Gracely presented a plenary session talk, Dr. Max served as Chair and Editor for the Pain Refresher Courses, Dr. Dionne was an invited speaker at two workshops, and numerous abstracts were presented by Drs. Ruda and Iadarola. Publications over the past year in respected peer-reviewed scientific journals attest to the Branch's continued scientific impact. Highlights of research findings by Branch investigators during the past year are presented below.

Antihyperalgesic effects of an AMPA/kainate receptor antagonist in humans: There is growing evidence from animal studies that neuropathic pain may be largely mediated by CNS excitation at glutamate receptors. There are three types of receptors in the central nervous system mediating glutamate's effects, NMDA, AMPA/kainate, and metabotropic receptors. Dr. Max and colleagues recently carried out the first administration in humans of an AMPA/kainate antagonist and showed that this class of glutamate receptor antagonists relieves pain and hyperalgesia produced in volunteers by intradermal administration of capsaicin. These initial results were extended in the past year to show that the AMPA/kainate antagonist LY293558 also relieves clinically relevant pain. A clinic trial treated 70 subjects with postoperative pain after third molar extraction with either placebo, high or low intravenous doses of LY 293558, or the standard analgesic ketorolac. The high dose of LY 293558 and the standard analgesic both significantly reduced pain compared to placebo. This is the first data to show that an AMPA/kainate receptor antagonist is effective in clinical pain. Although the magnitude of the effect is modest, preclinical studies of more selective compounds at a collaborating

pharmaceutical company suggest that the analgesic properties of the drug arise from selective blockade of GluR5 kainate receptors, which does not cause the sedation associated with mixed AMPA/kainate blockers. Investigators in the PNMB plan to study this next generation of compounds in humans when they are ready for human testing in 1-2 years.

Neonatal persistent pain produces plasticity in nociceptive neuronal circuits: Plasticity in the nervous system is increasingly recognized as a potential mechanism of chronic pain which persists after the initial stimulus or injury has been removed. Work in the Cellular Neuroscience Section this year has expanded insights into how this process may occur both anatomically and physiologically. Studies demonstrated a dramatic alteration of small diameter primary afferent spinal circuits in adult rats that experienced neonatal persistent pain. A rat model of inflammation and hyperalgesia examined the role of altered spinal neuronal circuitry on the release of Fos-protein-like immunoreactivity. The side of the spinal cord activated by peripheral inflammation consistently showed an increased number of cells expressing Fos-like immunoreactivity in the superficial laminae and neck of the dorsal horn, where nociceptive afferents typically terminate. A parallel study demonstrated physiologic changes in neonatal rats exposed to persistent painful stimulus including increases in background activity and responses to innocuous and noxious mechanical stimulation. The results of these studies demonstrate that altered spinal circuitry from persistent neonatal pain results in hyper-excitability of the spinal cord supporting the hypothesis of anatomical and physiologic plasticity. These observations may provide an explanation for the development and persistence of chronic pain states following tissue injury.

Genetics of chronic pain: Dr. Max initiated a program this past year to evaluate the genetic mechanisms of chronic pain. Use of a rodent model of neuropathic pain, autotomy following a sciatic nerve lesion, demonstrated wide variability among inbred strains in levels of pain behavior. Mapping is now underway to determine the loci of genes contributing to high and low pain behaviors following nerve injury. A parallel study is evaluating DNA from soldiers with war-related amputations and varying degrees of phantom limb pain to map microsatelite markers. Preliminary results indicate that one of these markers is significantly associated with the propensity to develop phantom limb pain. These unique observations may provide evidence for a genetic basis for some forms of chronic pain and will be extended in the next year to the study of the genetic contribution to chronic pain from a herniated lumbar disc.

Selective neurotoxins and gene transfer mechanisms of analgesia: Adequate control of cancer pain remains a significant clinical problem. To reduce side effects of treatment, spinal routes of administration have been used to achieve regional pain control with a reduced drug dose. Dr. Iadarola's lab demonstrated that administration of resiniferatoxin gives a long-term desensitization of C-fiber sensory neurons that mediate acute pain. When applied spinally, resiniferatoxin produced profound analgesia in animals that showed no restoration of pain sensitivity over 7 days. This route was 25 times more potent than the usual subcutaneous route of drug administration. This demonstration holds promise for use in humans suffering from intractable cancer pain without the significant adverse effects and lowered quality of life associated with high doses of opioids. Progress was also made this year towards developing in vivo gene transfer as a potential new means to treat chronic pain. Previous studies demonstrated in animals that a gene transfer approach using beta-endorphin incorporated into an adenovirus

was effective for selectively blocking pain. Development of the viral vector for human use was initiated this year and will be further developed for possible clinical use for chronic cancer pain.

Modulation of cytokine levels at the site of tissue injury alters postoperative pain: The microdialysis method has been used in oral surgery patients to demonstrate the release of pro-inflammatory cytokines locally and the relationship between altered levels and postoperative pain. Studies completed this year have demonstrated a profile of release of prostaglandin E_2 at the oral surgery site which is consistent with early release due to the activity of constitutive cyclooxygenase-1 and later release due to induction of cyclooxygenase-2. Measurements from biopsies collected prior to surgery and at the report of moderate pain demonstrate expression of cyclooxygenase at pain onset coincident with increases in prostaglandin E_2. Drugs which alter levels of the isoforms of cyclooxygenase result in parallel changes in cytokines and thromboxane B2 and pain supportive of a functional relationship between enzyme expression, release of pro-inflammatory cytokines and the perception of postoperative pain. These observations provide the rationale for the direct administration of an extended release formulation of a non-steroidal anti-inflammatory drugs which suppressed pain at doses lower that those usually administered orally. Local drug administration holds promise for increased analgesic efficacy by blocking the release of cytokines at the site of injury with much lower circulating drug levels, thereby decreasing exposure of potential targets of drug toxicity such as the GI tract and kidneys.

Imaging pain pathways in the humans: The human functional brain imaging program continues to define the neural networks that subserve pain, the interaction between these networks, and the functional abnormalities induced by chronic pain in select pain populations. This year has seen the expansion of the program to include the use of functional magnetic resonance (fMRI) which has greater temporal and spatial resolution than positron emission tomography (PET) and does not use ionizing radiation. Dr. Gracely has served as a collaborator on studies at Georgetown Medical Center assessing the symptom of tenderness in patients with fibromyalgia, a syndrome of unknown etiology involving widespread pain, tenderness and fatigue affecting an estimated 3.7 million people. As part of these investigations, a novel stimulator was developed that delivers controlled, rectangular-waveform pressure stimuli. The cerebral response to blunt pressure was evaluated by fMRI and demonstrated that few brain regions were responsive to innocuous stimulation, while increasing subjective pain intensity recruited additional brain regions associated with emotional and sensory-motor processing. A second study with fMRI demonstrated that stimulus intensities that evoked only faint pain in normals resulted in activation patterns in fibromyalgia patients similar to those caused by intense pain conditions in normal controls. This similarity in brain activation during subjectively equal conditions provides objective evidence that fibromyalgia involves an augmentation of pain sensitivity. An additional finding of these studies suggests that fibromyalgia is associated with altered supraspinal processing of pain information that involves both excitatory and inhibitory mechanisms.

Clinical evaluation of patients with failed TMJ implants: A series of patients with failed implants that were placed in the temporomandibular joint (TMJ) were evaluated to determine if any evidence of systemic disease exists and to develop strategies for pain management. A multidisciplinary evaluation and exhaustive laboratory testing documented a wide range of residual symptoms and systemic disturbances among patients. Preliminary findings are

consistent with a localized response to the implants which has been exacerbated by repeated surgical interventions. The most severely impaired patients appear to be suffering from significant iatrogenic injury due to repeated, failed surgeries indicating the need for comprehensive pain management at multidisciplinary pain clinics rather than repeated attempts at surgical intervention.

PAIN AND NEUROSENSORY MECHANISMS BRANCH
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Functional Genomics Unit

Ashok Kulkarni

FUNCTIONAL GENOMICS UNIT
1999

Our Unit continues to generate exciting research advances in the pursuit of basic scientific knowledge in dissecting pathways relevant to the disorders that affect craniofacial and dental systems as a result of genetic abnormalities. The Unit's primary research approach is centered on functional genomics. Since its inception, the key research work of the Unit has concentrated on the following studies:

Molecular Genetics of Development:
In the first set of studies, we have begun to delineate precise roles of Cyclin dependent kiase-5 (Cdk5) in neuronal phosphorylation to gain insight into its role in abnormal phosphorylation observed in a number of neurodegenerative disorders. Following cloning of Cdk5 gene, we generated mice deficient in Cdk5 expression which exhibit perinatal mortality associated with gross lesions in the brains and spinal cords. These mice lacked normal stratification of the neurons along with cerebellar defoliation, accumulation of neurofilaments in the neuronal cell bodies and ballooned motor neurons. Further studies revealed a typical inverted cortex in these mice indicating a special "cell autonomous" role of Cdk5 in neuronal migration. We have now generated Cdk5 "conditional" knockouts that exhibit abnormal gait, postures and reflexes indicating possible peripheral neuropathy.

In the second project, we chose to work on Fabry disorder because of its unique nature as a painful and fatal metabolic disorder and the challenges it presents in developing much needed therapeutic approaches. Following the cloning of the murine gene of alpha- galactosidase A, the gene involved in Fabry disease, we generated null mice, which exhibit lipid inclusions in the target organs typically seen in Fabry patients. Subsequent studies revealed that the aging of these mice accentuates and bone marrow transplantation ameliorates the phenotype of these mice indicating a potential for BMT as a therapy for some of the Fabry patients.

In the third project, we have begun to delineate in vivo role of dentin sialophospho protein gene (dspp) in dentinogenesis. We have cloned and begun to characterize the structure, regulation and functions associated with the dspp gene products. We have developed a transgenic animal model with a reporter gene (ß-galactosidase) under the control of 5.7 kb 5' flanking dspp and the analysis of these mice validates this promoter for tooth specific expression of the candidate genes and Cre transgene.

Molecular Genetics of Inflammation:
In this category of projects, we have begun to analyze the autocrine and endocrine roles of transforming growth factor beta-1 (TGF-ß1) and macrophage migration inhibitory factor (MIF) in immune dysregulation and inflammation. The initial findings from studies indicate ameliorating effects of MHC-1 deficiency in inflammatory responses in the absence of TGF-ß, potential for de novo TGF-ß1 gene therapy and also involvement of MIF in organogenesis.

FUNCTIONAL GENOMICS UNIT
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Immunopathology Section

Larry Wahl

IMMUNOPATHOLOGY SECTION
1999

Research in the Immunopathology Section focuses on the biological mediators and signal transduction pathways involved in the modulation of human monocyte functions that may contribute to the immunopathology associated with various disease states. Connective tissue destruction is associated with many diseases in which the monocyte/macrophage is a prominent cell. Since matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are believed to play a major role in the destruction and remodeling of connective tissue, a major emphasis has been placed on how these enzymes and inhibitors are regulated.

Regulation of MMP-2 activation has been of considerable interest in light of its potential role in cancer progression. The recently described membrane-type metalloproteinases (MT-MMPs) form a family of membrane-bound enzymes that, as one of their functions, activate MMP-2 through a mechanism that is thought to involve a trimeric complex with TIMP-2. Our recent studies have focussed on the potential role monocytes may have in the activation of MMP-2 through the production of MT1-MMP. This study demonstrated that while monocytes cells express low levels of mRNA for MT1-MMP, this enzyme is not found on the cell surface. In contrast, activation of monocytes resulted in enhanced synthesis of MT1-MMP and the translocation of MT1-MMP protein to the cell surface. Monocyte MT1-MMP was shown to be efficient at activating the MMP-2 produced by fibroblasts and cancer cell lines. The ability of the monocyte to activate the latent form of MMP-2 produced by other cell types appears to be related to the failure of the monocyte to produce appreciable amounts of TIMP-2. This allows free MT1-MMP on the surface of monocytes to bind the MMP-2/TIMP-2 complex produced by other cells, thereby activating the MMP-2 on an adjacent unoccupied MT1-MMP. In support of this conclusion is the ability of exogenous TIMP-2 to inhibit activation of MMP-2 by MT1-MMP. The findings in this study indicate that MT1-MMP on tumor associated macrophages may have a significant role in determining the progression of tumors.

Monocytes/macrophages may also play a crucial role in cardiovascular diseases due to their potential to develop into foam cells, a prominent cell type found in atherosclerotic plaques. It has been postulated that MMPs are involved in the rupture of plaques resulting in the ischemic events associated with stroke and myocardial infarction. We have initiated studies in several areas to address the functional role of monocytes in atherosclerosis. One project has focused on the role of oxidized LDL (oxLDL), LDL, and HDL in the regulation of monocyte MMP and cytokine production. Exposure of monocytes to oxLDL alone failed to induce MMP-1 and had no affect on the endogenous levels of MMP-9. However, oxLDL significantly enhanced MMP-1 production induced by LPS or the combination of TNF alpha and GM-CSF, but failed to influence the cytokine or LPS mediated increase in MMP-9. In contrast, LDL had no effect on the MMP production by LPS or cytokine activated monocytes. However, of particular interest was the ability of HDL to suppress the enhancement of MMP-1 by oxLDL. Thus the ratio of HDL to oxLDL may be important in the regulation of MMP-1 and possibly other MMPs in the atherosclerotic plaque.

In collaboration with scientists at the NHLBI we have initiated a second area of study in atherosclerosis. This project involves patient protocols in which estrogen or raloxifene is

administered to post-menopausal women with coronary artery disease and high cholesterol/LDL levels. The peripheral blood monocytes are obtained from these patients either before or after hormone treatment and evaluated for their production of MMPs. Additionally, these findings are compared with monocytes treated in vitro with these hormones or serum from hormone treated patients. Our in vitro findings demonstrated that low concentrations of estrogen or raloxifene caused a decrease in LPS induced MMP-1 production by control monocytes. In contrast the LPS mediated increase in MMP-9 was unaffected by these hormones. Of considerable interest was the finding that in vitro concentrations of raloxifene above 10 micro molar caused a substantial increase in MMP-1 production by LPS or cytokine treated monocytes. A concentration of 10 micro molar or higher is easily achievable in the patients given 60 mg of raloxifene. When monocytes obtained from these individuals given 60 mg of raloxifene were exposed to LPS, the cells of five out of six patients produced significantly higher levels of MMP-1 as compared to monocytes obtained when these individuals were on placebo. These findings suggest that the current clinically administered dose of raloxifene may elevate MMP-1 production by activated monocytes/macrophages, resulting in an increased risk for rupture of vulnerable atherosclerotic plaques.

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Matrix Metalloproteinase Unit

Henning Birkedal-Hansen

MATRIX METALLOPROTEINASE UNIT
1999

The Matrix Metalloproteinase Unit explores the distal regulatory mechanisms which enable cells to locally dissolve and degrade the extracellular matrix. Specifically we seek to define the role of MMPs and their inhibitors in this process. A body of evidence suggests that MT1-MMP, Gelatinase A and TIMP-2 play an important role in initiating the catalytic activation of MMPs on the cell membrane, at least in vitro, and raises the question whether this mechanism serves as a common and perhaps universal MMP activation pathway. Our recent efforts have targeted this process for investigation

Inactivating Mutation of the MT1-MMP Gene

To assess the biological function of MT1-MMP, we disrupted the murine gene by homologous recombination and generated mice with a null phenotype.

MT1-MMP Deficient Mice are Viable but Display Severe Runting, Wasting, and Increased Mortality.

Neonate MT1-MMP-deficient mice could not be distinguished visibly from heterozygous and wild type littermates at birth, but growth impairment (smaller body size and weight) became evident as early as 5 days later. One third of mutant animals died from wasting before weaning and surviving mutant mice gained little weight over the next few weeks and after day 50 experienced progressive wasting, patchy hair loss, reduced mobility, kinking of the wrist, and hyperlordosis/hyperkyphosis. The mutant mice showed no signs of sexual maturation and most of the animals died between day 50 and 90.

MT1-MMP Deficiency Causes Skeletal Dysplasia, Arthritis, Severe Osteopenia, and Generalized Soft Tissue Disorders

MT1-MMP deficient mice gradually developed a severe skeletal phenotype short snout, orbital protrusions, hypertelorism, and dome-shaped skull with bulging parietal and interparietal regions became obvious at day 5. Membranous ossification of calvarial bones was delayed and suture closure was never completed. Beginning at day 5, the limb bones appeared shorter than in control littermates and grew to approximately 65% of the length of controls by day 45. Osteopenia became increasingly apparent and bone mass was severely reduced in animals aged 40 days or older. Concurrently, MT1-MMP deficient mice developed severe generalized arthritis. All joints showed overgrowth of a hypercellular, vascularized synovial tissue and destruction of articular cartilage, resulting in ankylosis. TRAP-positive, osteoclast-like giant cells, were prominent within articular and periarticular soft tissues. Tendons, ligaments, synovial capsules, musculotendinal junctions and septal/fascial structures associated with skeletal muscle, all displayed increased cell proliferation and vascularity, and became increasingly fibrotic. Older animals developed progressive fibrosis of the dermis and of hair follicles, which coincided with hair loss.

MT1-MMP Deficiency Impairs Collagenolytic Activity and Osteogenic Potential of Osteoblasts

Since our findings showed that bone formation was markedly reduced in MT1-MMP deficient animals, we isolated clonogenic osteoprogenitor cells (CFU-F) from the bone marrow of mutant mice and normal littermates, and probed their ability to form bone in a transplantation assay. CFU-F were not quantitatively deficient in the bone marrow of mutant mice. However, when culture-

expanded populations of marrow-derived osteogenic cells from mutant were loaded onto either (denatured) insoluble collagen-type-I-based (Gelfoam™) or hydroxyapatite-based carriers they failed to form bone unlike wild type cells. The Gelfoam™ matrix was efficiently degraded and replaced by bone and marrow in ossicles formed by wild type cells, whereas a significant fraction of the collagenous carrier material remained undegraded in ossicles formed by mutant cells. We concluded that MT1-MMP-deficient marrow stromal cells possess two seemingly related defects: impairment of osteogenic capacity and impairment of collagenolytic/gelatinolytic activity.

MT1-MMP Deficient Skin Fibroblasts are Incapable of Degrading Type I collagen Matrices In Vitro.

Because of the apparent inability of MT1-MMP deficient marrow stromal cells to degrade the collagenous Gelfoam™ matrix, we sought to verify that MT1-MMP deficient cells were deficient in collagenolytic activity. Skin fibroblasts isolated from newborn mice were seeded in a pellet on a reconstituted type I collagen fibril film and incubated in serum-free medium. Cells derived from wild type or heterozygous mice readily degraded the underlying collagen film particularly when stimulated with TNFα/IL-1β. Cells from MT1-MMP deficient littermates, however, completely failed to degrade the collagen matrix demonstrating that MT1-MMP deficiency imparts a severe defect in collagenolytic activity and that degradation of type I collagen fibrils in this system is absolutely dependent on MT1-MMP.

Inactivating Mutation of the TIMP-2 Gene

Disruption of the TIMP-2 gene by deletion of exons 2 and 3 resulting in ≥99% reduction of TIMP-2 activity gave rise to seemingly minor phenotypic changes, but further analysis unveiled significant and profound differences between wildtype and mutant mice. Cells derived from mutant mice showed reduced ability to activate Gelatinase A (MMP-2) and this activity was restored by exogenous TIMP-2. The role of TIMP-2 was further tested in a murine tumor model in which the TIMP-2 mutant genotype was bred into a transgenic mice carrying middle T antigen gene under control of the mouse mammary tumor virus promoter. Primary breast tumors develop in virgin females by 2 months of age which then, by a random genetic event, metastasize to the lungs at high frequency by 4 months of age. In this model, mice homozygous for the mutant TIMP-2 locus had much lower levels of lung metastasis than those with wildtype TIMP-2 loci. We also noticed that matrix-associated Gelatinase A is activated in extracts of normal lungs, but remains latent in extracts of lungs from TIMP-2 deficient mice, providing evidence for a role of TIMP-2 in regulation of proGelatinase A activation *in vivo*. While the mechanism remains to be understood in greater detail, these findings suggest that activation of proGelatinase A activation may play a significant role in regulating mammary tumor metastasis.

Regulation and Activation of Collagen Degradation

In search of enzymes and inhibitors which regulate and catalyze the metabolic degradation of extracellular matrix, we pursued a strategy which allows us to monitor the dissolution of a proteolytically resistant, single-component matrix (collagen type I fibrils) by live cells. Transfection (reconstution) experiments performed with COS and CHO cells which do not degrade collagen fibrils permitted us to define the minimal requirements for generation of a collagenolytic phenotype. These experiments showed that furin, MT1-MMP and either Gelatinase A or collagenase-3 are minimally required and/or rate limiting. Since MT1-MMP contains a furin cleavable activation site, we surmise that the sequence of events includes (but may not be limited to) furin activation of MT1-

MMP followed by MT1-MMP activation of Gelatinase A or collagenase-3. Both Gelatinase A and collagenase-3 are capable of cleaving reconstituted fibrils of type I collagen.

Inactivating Mutation of the Enamelysin Gene

The MMP unit also conducted studies on enamelysin (MMP-20) which appears to be the only MMP specific for tooth development. We isolated, and completed the characterization of, the 65 kilobase mouse enamelysin gene. A targeting strategy was devised which leads to deletion of exons 4 and 5 (containing the zinc-binding site of the catalytic domain) in mouse HM-1 embryonic stem cells through dual selection for the process of homologous recombination in HAT/gancyclovir medium. A targeting efficiency of ~30% was obtained. Seven targeted clones were used in blastocyst injections giving rise to high percentage chimeric offspring from at least 4 separate clones. Chimeras were backcrossed to obtain germline transmission of the targeted MMP-20 locus to F1 animals in both the outbred (C57bl/6 x 129/ReJ) and inbred (129/ReJ) backgrounds. Germline hemizygous animals were bred to homozygosity.

Histomorphologic analysis suggests that expression of functional enamelysin may not be critical during the process of tooth development. Homozygous mutant mice have seemingly normal teeth. No obvious histological differences can be observed between sections of teeth from 2 week old wildtype and homozygous mutant mice stained with H & E. Both ameloblast and odontoblast layers appear normal. The degree of mineralization and residual enamel matrix within the enamel layer at this timepoint look comparable.

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Molecular Structural Biology Unit

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MOLECULAR STRUCTURAL BIOLOGY UNIT
1999

The principal research goal of the MSBU is to elucidate the structure and dynamics of proteins, RNAs and associated molecules at the molecular level in order to provide a basis for understanding function. The main research tool used in this work is high resolution, multidimensional, nuclear magnetic resonance (NMR) spectroscopy. Three projects are currently active (1) HIV-1 protease, as either the free enzyme or bound to a high affinity protease inhibitor (2) the RNA binding protein S4, either free or bound to a target RNA molecule (3) MAP-30, an anti-HIV/anti-tumor protein. Progress that has been made during the past year in relating the structure and dynamics of these molecules with their functions is discussed below.

HIV-1 protease

Although potent inhibitors of the HIV-1 protease are currently used in therapies against AIDS, these drugs have a limited effect, due to selection of drug-resistant variants of the virus. Hence, understanding inhibitor-protease interactions at the molecular level remains of great interest. Analyses of the interactions between the protease and inhibitors have been made based upon crystal structures. These studies together with NMR studies of the protease in solution have indicated that dynamics of the protease plays an important role in its interactions with inhibitors. We have compared the molecular dynamics of a fully active, but stable protease construct (Q7K, L33I, L63I) free and bound to the potent inhibitor, DMP323. Our initial studies showed that fast internal motions (on the sub-nanosecond time scale), having significant angular amplitudes, take place for NH bonds of flap residues G48 through F53. We suggest that these motions are a consequence of a rapid dynamic equilibrium between various hydrogen bonded beta-hairpin (semi-open) structures, seen in crystal structures of the free protease. Such rapid motions are not observed in the inhibited molecule, because the flaps form part of a hydrogen bonded network involving the inhibitor, and motion is restricted to slow flips of the I50-G51 peptide plane. Although these observations show that the flaps are highly dynamic structures in the free protein, molecular dynamics calculations indicate that the flaps cannot change from hydrogen bonded, semi-open, beta-hairpin structures to open structures on the nanosecond time scale (Rick et al., Proteins, 1998, *32*, 7). This has prompted us to develop a novel method to study slow (on the ms-μs time scale) backbone motions in the protease, using complementary measurements of amide ^{15}N and ^{1}H transverse relaxation times. These measurements revealed that in the free protease, residues G48 through I54 undergo slow conformation exchange. We suggest that the fast motions of the flaps involve conformation fluctuations among the semi-open hairpin conformations, whereas the slow motions reflect the dynamic equilibrium between the semi-open and fully open flap conformations, and permit entry of substrates and inhibitors to the active site of the protein. KNI-529 is a potent inhibitor (K_i ca. 10^{-9} M) of the protease. Because KNI-529 is asymmetric, and is in slow exchange with the protein, the protease monomers have distinct NMR spectra when the protein is bound to the inhibitor. Remarkably, the NOESY spectrum of the protease/KNI-529 complex shows a cross peak between the amide proton in residue i (in monomer 1) and the amide proton in residue i+100 (the equivalent residue in monomer 2), regardless of the distance between the two protons. These distance independent cross peaks arise because the orientation of the inhibitor, relative to the protein, reverses, and exchanges the chemical shifts of amide protons i and i+100. The rate of monomer exchange, k, was found to increase from ca. 0.2 s^{-1} to 6 s^{-1} upon increasing temperature from 25 to 45°C. Our analysis of

the temperature dependence of k showed that the inhibitor flips without dissociating from the protein, with the rate of flap opening of ca. 1 s^{-1} at 35°C. It is noteworthy that the rate of flap opening is ca. three to four orders of magnitude slower in the presence of the KNI529 than in the free protease. Currently we are investigating slow conformational fluctuations of protease sidechains, and have devised novel experiments to characterize such conformational changes in the protease flaps and in the substrate/inhibitor binding sites.

Structures of the ribosomal proteins S4delta41 and S4.

Prokaryotic ribosomal protein S4 binds to 16S rRNA to nucleate assembly of the small subunit of the ribosome. Mutations in S4 affect translational accuracy and antibiotic resistance. Like many ribosomal proteins, S4 also binds to its own messenger RNA to regulate its own translation. Strikingly, the RNA targets for S4 have been reduced to disparate structures, the mRNA site includes about 100 nucleotides in a pseudoknot, while the rRNA site includes about 460 nucleotides in hairpins. As a step toward understanding how S4 recognizes such disparate targets, we have solved the structure of its RNA binding domain (S4d41, 159 residues) by solution NMR spectroscopy. S4delta41 has a novel fold with two distinct subdomains, one comprised of four helices, the other comprised of three helices and a five-stranded antiparallel beta-sheet. The cleft between the subdomains is lined with positively charged side chains from both subdomains, suggesting a likely RNA-binding site. Comparison of the average NMR structure with the independently solved crystal structure reveals the same organization for each subunit but the relative orientation of the subunits with respect to the long axis of the molecule was not well defined by the NMR restraints. We therefore refined the NMR structure by including dipole coupling restraints derived from NH couplings measured in two different liquid crystal media. The subdomain orientation of the refined S4delta41 structure is well-defined in solution and within 10 degrees of that observed in the crystal structure. This is among the first demonstrations of the power of dipolar couplings to establish subdomain orientations in solution structures. Intact S4 binds to mRNA about ten-fold better than S4delta41 and we have began an investigation of the structure of the full-length protein. The intact protein was overexpressed in *E. coli*. It was purified to homogeneity using a modified procedure that removed protease contaminants shown to digest the N-terminal segment. Using samples enriched in ^{15}N, NMR relaxation studies were performed which showed that this N-terminal segment is highly mobile in solution, suggesting that this segment has little organized secondary structure. To understand these results more fully, samples were prepared enriched in both ^{15}N and ^{13}C, and the backbone atoms of the entire protein were assigned using triple resonance experiments. The signal assignments revealed that the N-terminal segment populates several conformers in solution. These conformations are in slow exchange as a result of cis-trans isomerism involving the five proline residues in the segment. The signal assignments for residues in the remainder of the protein were essentially unchanged from S4delta41, with the exception of residues near the junction with the N-terminal segment. Scalar couplings between amide and alpha protons were measured using the HNHA experiment, and the carbon chemical shifts were analyzed according to chemical shift index. Both of these analyses also suggested that the N-terminal segment has little secondary structure. ^{15}N and ^{13}C NOESY-HSQC spectra were also acquired, and few close contacts were observed for protons of the N-terminal segment. However, some short-range contacts were found for residues near prolines, and these are being investigated further, as they may indicate the presence of some localized structure. The NOESY evidence further suggests that the structure of the remainder of the protein is very similar to that of S4d41. Now that we

have completed the assignments of intact S4 and characterized its structure, experiments are underway (a) to determine if the N-terminal 41 residues assume an ordered structure when S4 binds to RNA and (b) to identify the residues of S4 and S4d41 that are involved in binding RNA.

Structure/function study of the anti-HIV/anti-tumor protein Map30

MAP30 is a ribosome inactivating protein (RIP) derived from bitter melon (*Momordica charantia*) a Chinese medicinal plant. RIPs are of wide interest because they are cell toxins with potent anti-viral/anti-tumor activities. In addition to their well-established RNA N-glycosylase (RNG) activity against 28S rRNA, MAP30 and other RIPs have been reported to have DNA enzymatic activity. The mechanism(s) of their anti-viral/anti-tumor activities are not fully understood, and it has been claimed that their DNA enzymatic activity is due to nuclease contaminates. In addition it has been reported that MAP30 and gelonin inhibit HIV-1 integrase, an activity not reported for other RIP proteins. We have solved the high resolution solution structure of MAP30 in order to obtain a structural basis for understanding RIP functions. This structure was determined from a total of 3500 distance, dihedral angle and dipolar coupling restraints, and, at 30kDa, MAP 30 is one of the largest proteins whose solution structure has been determined without a prior X-ray structure. The solution structure of MAP30 is similar to the crystal structures that have been determined for other RIPs. This observation is not surprising in view of the fact that RIPs have homologous amino acid sequences. It does however suggest that there is no obvious structural basis for the disparate activities that have been reported for RIP family members. With this in mind, we hypothesized that some reported MAP30 activities, such as inhibition of HIV integrase might be due to the fact that MAP30 competes with integrase for a common DNA substrate. This hypothesis was supported by chemical shift perturbation data that showed that amino acid residues in the neighborhood of the RNA glycosylase active site specifically interact with the LTR DNA. We next used $NaBH_4$ to trap an imine cross-link between MAP30 and LTR RNA. This observation provides evidence that after binding, MAP30 depurinates DNA and acts as DNA lyase. Further NMR studies showed that metal ions needed for these activities facilitate DNA substrate binding by shielding the negatively charged protein and DNA surfaces, rather than by acting directly as catalytic metal ions. The sidechain of strictly a conserved Trp residue (W190 in MAP30) is observed on the protein surface, adjacent to the N-glycosylase active site, in all known RIP structures. Because we have shown that the LTR DNA binds at this site we postulate that DNA is also depurinated at this active site. Apurinic DNA is expected to have greatly enhanced binding affinity towards Trp190, based upon studies of the binding of Lys-Trp-Lys, and related peptides, to intact and depurinated DNA. Binding to Trp190, brings the apurinic DNA site close to a lysine sidechain in Map30, and in PAP and in gelonin, two other RIPs reported to have DNA deglycosylase/ap lyase (DGAL) activity. This enables the amino group of the lysine to function as an attacking nucleophile, in a manner similar to amino groups that have been identified as attacking nucleophiles in DNA DGAL repair enzymes, and in peptides that cleave DNA at apurinic sites. It is noteworthy that the ricin A chain, which contains only two Lys residues, neither near the conserved Trp, has been reported to depurinate DNA, but shows no evidence of ap lyase activity. We therefore suggest that RIPs utilize a common active site to depurinate both DNA and 28SrRNA. Furthermore, we propose that, following depurination of DNA, the apurinic DNA site interacts with the conserved Trp sidechain, thus facilitating nucleophilic attack by a nearby Lys sidechain. Hence, in our model, the depurnation and cleavage reactions take place at distinct, but contiguous, sites. Note,

apurinic DNA cleavage activity involving other surface aromatic sidechains, close to lysine amino groups, is a possibility.

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